IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: O'Brien et al	§	**ART UNIT:** 1642
FILED: October 20, 1999	§	
	§	**EXAMINER:**
SERIAL NO.: 09/421,213	§	**Harris, A.**
	§	
FOR: TADG-15: An Extracellular Serine Protease Overexpressed In Carcinomas	§	**DOCKET:** D6064CIP

MS Appeal Brief
Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313

TRANSMITTAL OF APPEAL BRIEF

Dear Sir:

Enclosed please find three originals of the Appeal Brief for the above-referenced patent application.

The Commissioner is hereby authorized to charge Deposit Account No. 07-1185 in the total amount of $165 for the appeal fee and any additional fee that may be required. Please credit any overpayment or debit any underpayment to Deposit Account 07-1185.

Respectfully submitted,

Date: Oct 28, 2003

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ATTENTION: Board of Patent Appeals and Interferences

APPELLANT'S BRIEF

This Brief is in furtherance of the Notice of Appeal filed in this case on July 28, 2003. The fees required under 37 C.F.R. §1.17(f) and any other required fees are dealt with in the accompanying TRANSMITTAL OF APPEAL BRIEF.

In accordance with 37 C.F.R. §1.192(a), this Brief is submitted in triplicate.

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I. REAL PARTY IN INTEREST

The real party in interest is the University of Arkansas for Medical Sciences.

II. RELATED APPEALS AND INTERFERENCES

Appellant is aware of no other appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF THE CLAIMS

Originally claims 1-52 were filed with this Application. Claims 1-21 and 25-52 were withdrawn from consideration. The pending claims 22-24 are being appealed of which claim 22 and 24 are independent claims.

IV. STATUS OF AMENDMENTS

No amendment was made subsequent to the final rejection mailed November 5, 2002. All pending claims are shown in Appendix A.

V. SUMMARY OF THE INVENTION

The present invention discloses cloning and sequencing of a novel serine protease, Tumor Antigen Derived Gene-15 (TADG-15) protein which is overexpressed in ovarian carcinoma (page 13, line 20 to page 14, line 10). Nucleotide sequence and amino acid sequence for TADG-15 are disclosed in SEQ ID NO. 1 and SEQ ID NO. 2 respectively (Figure 2). TADG-15 transcript expression was elevated in various ovarian tumor cells with different stage and histological sub-type, but it was not present at detectable levels in normal ovaries (Example 11; Figure 6A and B; Table 2). Expression of TADG-15 was also observed in carcinomas of the breast, colon, prostate and lung (Figure 8). Antibody staining of tumor cells confirms the presence of the TADG-15 protease in the cytoplasm of

ovarian cancer cells (Figure 10). No antibody staining was detected in normal ovarian epithelium or stromal cells (Figure 10A). These data suggest that the TADG-15 gene and its translated protein will be a useful marker for the early detection of ovarian carcinoma and TADG-15 can be a target for therapeutic intervention (page 72, line 19 to page 73, line 4).

VI. ISSUES

35 U.S.C. §103

Whether claims 22-24 are obvious over **GenBank Accession Number W22987** in view of **Lerner** under 35 U.S.C. §103(a).

VII. GROUPING OF CLAIMS

The rejected claims do not stand or fall together. Applicant submits that claims 22-23 are patentably distinct from claim 24 because a kit for detecting Tumor Antigen Derived Gene-15

(TADG-15) protein is patentably distinct from an antibody specific for Tumor Antigen Derived Gene-15 (TADG-15) protein.

VIII. ARGUMENTS

Rejection Under 35 U.S.C. §103

In the Advisory Action mailed December 31, 2002, the Examiner maintained that claims 22-24 were rejected under 35 U.S.C. §103(a) as being unpatentable over **GenBank Accession Number W22987** (1997) in view of **Lerner** (1982). Applicant respectfully disagrees and requests that the Board reverse this rejection.

As it is indicated above, claims 22-23 and claim 24 do not stand or fall together. Applicant submits that these two groups of claims are separately patentable because the antibody of claim 24 can be used in a materially different process of using the antibody as stated in claims 22-23 (M.P.E.P. 806.05(h)). For example, the antibody of claim 24 can be used to purify TADG-15 by affinity

chromatography instead of being used to detect the presence of TADG-15 as claimed in claims 22-23.

TADG-15 is a protein with 855 amino acids. In contrast, **GenBank Accession Number W22987** teaches a protease with 241 amino acids. The sequence of **GenBank Accession Number W22987** is identical to a portion of TADG-15. The Examiner acknowledges that **GenBank Accession Number W22987** does not teach purified antibodies which specifically bind specified polypeptides, nor a kit comprising said antibodies (page 5, Office Action mailed June 29, 2000). The Examiner contends, however, **Lerner** teaches that antibodies can be generated against peptides of at least 15 amino acids, and representative of virtually any part of the surface of a protein can elicit antibodies reactive with the native molecules. Accordingly, the Examiner argues that it would have been prima facie obvious to one of ordinary skill in the art to select specific regions of the polypeptide of **GenBank Accession Number W22987** or the entire fragment in order to produce antibodies reactive with said TADG-15 protein as taught in **Lerner** (page 5, Office Action mailed June 29, 2000).

The antibody generated according to the Examiner's methodology would bind to both the protease described in **GenBank Accession Number W22987** and TADG-15. In contrast, Applicant submits that claims 22-24 recite an antibody specific for TADG-15 protein, not an antibody that would bind to both the protease of **GenBank Accession Number W22987** and TADG-15. The Examiner, however, rejects claims 22-24 based on the assertion that the term "specific" is not the same as exclusive, and asserting that an antibody raised against protease of **GenBank Accession Number W22987** would fit the criterion listed in Applicant's claims (page 4, Office Action mailed July 13, 2001). Applicant respectfully disagrees.

Arugment Concerning Claims 22-23

Claims 22-23 are drawn to a kit for detecting Tumor Antigen Derived Gene-15 (TADG-15) protein, the kit comprising an antibody specific for TADG-15 protein. The present specification teaches that TADG-15 was expressed in ovarian tumor cells but not in normal ovary as detected at the transcript and protein levels (Figures 6, 10; Table 2). TADG-15 was also expressed in carcinomas of the breast, colon, prostate and lung (Figure 8). Thus, one of

ordinary skill in the art would readily recognize the utility of specific detection of TADG-15 protein as a marker for ovarian and other tumor cells.

In order to distinguish between different cell types, one of ordinary skill in the art would require that the antibody specific for the protein marker does not cross-react with other proteins. Otherwise, cross-reaction with other proteins would lead to false positive results and diminishes the utility of the detection assay. Accordingly, in the context of the present invention, the utility of the detection kit lies in its ability to specifically detect TADG-15 in normal and tumor cells. Thus, the issue is whether one of ordinary skill in the art would follow the Examiner's methodology to generate an antibody that is useful as a component of a kit used to detect TADG-15 protein as a marker for tumor cells.

Applicant submit that one of ordinary skill in the art would not follow the Examiner's logic and methodology to generate a TADG-15-specific antibody. The antibody generated according to the Examiner's methodology would cross-react between W22987 and TADG-15 and would not be, therefore, specific for TADG-15. Thus, one of ordinary skill in the art would not recognize the antibody

generated according to the Examiner's proposed method as useful for specific detection of the marker protein TADG-15.

In order to obtain TADG-15-specific antibody that would be useful in detecting TADG-15 protein specifically, one of ordinary skill in the art would generate an antibody against a sequence which is unique to TADG-15. Such a unique sequence can be readily obtained by comparing TADG-15 sequence with other published sequences using a number of readily available softwares. One of ordinary skill in the art would not use a sequence which is common to the TADG-15 protein and other proteins (such as the sequence described by **GenBank Accession Number W22987** suggested by the Examiner) to generate an antibody specific for TADG-15 protein. Such an antibody is cross-reactive rather than specific for the TADG-15 protein. The kit recited by claims 22-23 requires an antibody specific for TADG-15 protein, not an antibody that would cross-react with TADG-15 and other proteins. A cross-reactive antibody would have little utility in detecting TADG-15 as a marker for tumor cells.

Hence, the combined teaching of **GenBank Accession Number W22987** and **Lerner** does not provide a person having ordinary skill in this art with the requisite expectation of successfully producing Applicant's claimed invention. The invention as a whole is not *prima facie* obvious to one of ordinary skill in the art at the time the invention was made. Accordingly, Applicant respectfully requests that the rejection of claims 22-23 under 35 U.S.C. §103(a) be withdrawn.

Arugment Concerning Claim 24

Claim 24 recites an antibody specific for TADG-15 protein. The Examiner contends that that an antibody raised against protease of **GenBank Accession Number W22987** would fit the criterion listed in Applicant's claims (page 4, Office Action mailed July 13, 2001). Applicant respectfully disagrees.

An antibody raised against protease of **GenBank Accession Number W22987** would bind to both protease of **GenBank Accession Number W22987** and TADG-15. Such an antibody would fit the limitation of claim 24 if claim 24 simply recites an antibody that binds to TADG-15. Claim 24, however, is not drawn

to an antibody that simply binds TADG-15 protein. Claim 24 is drawn to an antibody specific for TADG-15 protein. An antibody raised against protease of **GenBank Accession Number W22987** is a cross-reactive antibody that binds to both protease of **GenBank Accession Number W22987** and TADG-15. A cross-reactive antibody is not a specific antibody for TADG-15. Thus, an antibody raised against protease of **GenBank Accession Number W22987** does not meet the limitation of claim 24.

As discussed above, in order to obtain a TADG-15-specific antibody, one of ordinary skill in the art would generate an antibody against a sequence which is unique to TADG-15. Such a unique sequence can be readily obtained by comparing the TADG-15 sequence with other published sequences using a number of readily available softwares. One of ordinary skill in the art would not use a sequence which is common to TADG-15 protein and other proteins (such as the sequence of the protease of **GenBank Accession Number W22987** suggested by the Examiner) as an immunogen to generate an antibody specific for TADG-15 protein.

In view of the above remark, the combined teaching of **GenBank Accession Number W22987** and **Lerner** does not

provide a person having ordinary skill in this art with the requisite expectation of successfully producing Applicant's claimed antibody. The invention as a whole is not *prima facie* obvious to one of ordinary skill in the art at the time the invention was made. Accordingly, Applicant respectfully requests that the rejection of claim 24 under 35 U.S.C. §103(a) be withdrawn.

Respectfully submitted,

Date: Oct 28, 2003

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CLAIMS ON APPEAL

22. A kit for detecting Tumor Antigen Derived Gene-15 (TADG-15) protein, comprising:

an antibody, wherein said antibody is specific for TADG-15 protein.

23. The kit of claim 22, further comprising:

means to detect said antibody.

24. An antibody, wherein said antibody is specific for Tumor Antigen Derived Gene-15 (TADG-15) protein.

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ALIGNMENTS

RESULT 1
ID W22987 standard; Protein; 241 AA.
AC W22987;
DT 08-OCT-1997 (first entry)
DE Human serine protease 67 (SP67).
KW Human; colon carcinoma; COLO 201; cell line; serine protease; SP67;
KW screening; inhibitor; treatment; disease.
OS Homo sapiens.
PN J09149790-A.
PD 10-JUN-1997.
PF 24-JUL-1996; 212196.
PR 29-SEP-1995; JP-275105.
PA (SUNR) SUNTORY LTD.
DR WPI; 97-357902/33.
DR N-PSDB; T79128.
PT Human colon carcinoma derived serine protease(s) SP59, SP60 and SP67
PT - useful to screen for specific inhibitors, e.g. to search for, or
PT study agent for treatment of various diseases
PS Claim 1; Pages 12-13; 16pp; Japanese.
CC The present sequence is the human colon carcinoma COLO 201
CC cell line derived serine protease 67 (SP67), which can be used to
CC screen for specific inhibitors, e.g. to search for, or study an
CC agent for the treatment of various diseases.
SQ Sequence 241 AA;

Query Match 28.3%; Score 1808; DB 1; Length 241;
Best Local Similarity 99.6%; Pred. No. 8.25e-161;
Matches 240; Conservative 0; Mismatches 1; Indels 0; Gaps 0;

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Db      1 VVGGTDADEGEWPWQVSLHALGQGHICGASLISPNWLVSAAHCYIDDRGFRYSDPTQWTV 60
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Qy    795 SGGVDSCQGDSGGPLSSVEADGRIFQAGVVSWGDGCAQRNKPGVYTRLPLFRDWIKENTG 854

Db    241 V 241
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REVIEW ARTICLE

Tapping the immunological repertoire to produce antibodies of predetermined specificity

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We understand the structure of antibodies in detail, but know little about the molecular basis of the immunogenicity of proteins. Recent experiments have shown that chemically synthesized peptides representative of virtually any part of the surface of a protein can elicit antibodies reactive with the native molecule. Such peptides can serve as synthetic vaccines, and antibodies, useful in the study of changes in protein structure, can be generated. As these antibodies react with regions of the protein known in advance to the experimenter, they can be said to be of predetermined specificity.

THE immune system of a mammal is one of the most versatile systems in the biological kingdom as probably greater than 1.0×10^7 antibody specificities can be produced[1]. Indeed, much of contemporary biological and medical research is directed toward tapping this repertoire. As it is so vast, it might appear to be a relatively simple matter to produce antibodies of a particular specificity but until recently this was not the case because of two essential complications. The first problem is that serum antibodies consist of a mixture of molecules of diverse reactivity. As such they are useful for studying whole proteins but an understanding of fine specificity is difficult if not impossible. The development by Köhler and Milstein of the hybridoma methodology has solved this problem by making it possible to obtain in pure form antibodies of a single specificity from those induced during an immune response[2] but this left a second problem in that during an immune response to an intact protein antibodies are only produced against a very limited set of determinants within the protein molecule. This problem is the one to be discussed here.

Limited response to intact proteins

The key to eliciting antibodies of predetermined specificity is an understanding of what constitutes an immunogenic determinant on a protein. Whereas the term an antigenic determinant simply reflects the ability of a region in a protein to bind to antibodies, an immunogenic determinant is a region capable of inducing antibody. (Here we further define an immunogenic determinant as one that induces antibody reactive with the native protein.)

Previous studies on the nature of antigenic determinants, mostly following immunization with intact proteins, have led to two fundamental conclusions (reviewed in ref. 3, see also refs 4–21). The first is that during an immune response to a native protein, antibody reactivity is confined to only a few regions of the molecule. Studies on enzymatically fragmented proteins suggested that most globular proteins contain fewer than five antigenic sites; as a rough rule, about one site per 5,000 daltons of protein. The second conclusion is that antigenic determinants are dependent on tertiary conformation and are often constructed from discontinuous regions of the protein chain brought into proximity by folding of the molecule. These determinants are called 'conformational' or 'discontinuous'. Thus, by the mid-1970s, a picture of the antigenic profile of a protein had emerged. The determinants are few in number and largely dependent on native conformation (Fig. 1). However, throughout these studies it was tacitly assumed that antigenicity and immunogenicity are equivalent; in other words, the number of antigenic determinants of an intact protein was presumed to set a limit on the number of protein fragments which would carry immunogenic determinants.

The repertoire can be tapped

Needless to say, the above concepts did not bode well for a general method of producing antibodies reactive with most regions of a protein molecule. Our own interest in the problem followed an experiment we carried out to detect a protein potentially encoded by the Moloney leukaemia virus genome. During our sequencing of this genome, we found an open reading frame whose coding capacity did not fit with the known biochemistry of the viral proteins, a problem we have called genotype in search of phenotype. We decided to synthesize chemically a peptide from within the protein predicted by the nucleotide sequence, raise an antibody to it, and see if this antibody reacted with protein(s) in infected cells. Because of uncertainties as to how or if RNA splicing might be taking place, we synthesized a peptide potentially encoded by the 3′ end of the reading frame, and thus representative of the C-terminus of the putative protein. Indeed, the anti-peptide antibody precipitated a protein from infected but not normal cells[22]. Such an approach could be very useful as more and more DNA sequences were generated, but it was far from proven as a general methodology. We had studied the C-terminus of a protein and it seemed possible that the method might be useful only in detecting the ends of proteins. It was thought that an untethered C-terminus of a protein was relatively free to move and could be thought of as a kind of hapten carried by the rest of the molecule, a situation which we could have fortuitously duplicated when we coupled the peptide to the carrier protein for immunization.

To test the generality of the method we carried out a study on a protein of known structure. We chose the influenza virus haemagglutinin (HA) because the complete nucleic acid sequence of its gene is available[23] and its crystallographic structure is known at high resolution[24]. A series of peptides corresponding to 75% of the HA1 chain were chemically synthesized (we have now synthesized additional peptides so that the entire span of the molecule is represented) and antibodies made to each of the peptides. Antibodies to almost all (18 of 20) peptides reacted with the native molecule[25]. Because in its folded state the HA molecule displays a number of conformations including α-helix, random coil and β-sheets, it is clear that the ability of the anti-peptide antibodies to react with the intact structure is independent of any particular conformation or location in the molecule[25]. Probably the only requirement for selecting an immunogenic peptide is that a part of the sequence be located on the surface of the molecule so as to be available to antibody. In Fig. 2*b*, we show the portion of the surface of the HA molecule against which we have made antibodies using chemically synthesized peptides as immunogens. In Fig. 2*a*, we show those areas of the molecule thought to be immunogenic during viral infection or immunization with intact virus or purified viral

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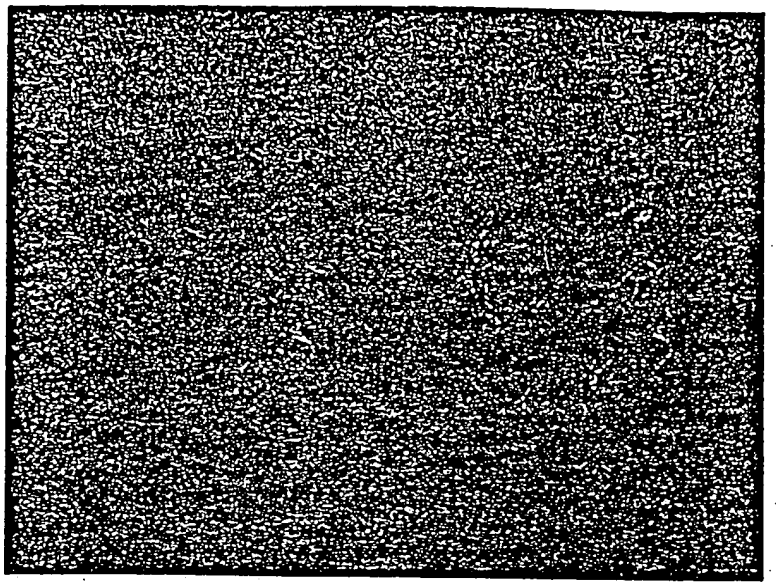

Fig. 1 Exposed surfaces of antigenic sites (shown in pink) of hen's egg-white lysozyme. Stereo projections showing the limited number of antigenic sites in the lysozyme molecule and their discontinuous ('conformational') nature. The data are based on Atassi and Lee[71] using the structure of Blake *et al.*[72] and Imoto *et al.*[73]. Exposed surfaces based on α-carbon positions using molecular surface computer program of Connolly[74]. The three antigenic sites are constructed by the spatially contiguous residues as follows: (1) Arg 125, Arg 5, Glu 7, Arg 14 and Lys 13; (2) Trp 62, Lys 97, Lys 96, Asn 93, Thr 89 and Asp 87; (3) Lys 116, Asn 113, Arg 114, Phe 34 and Lys 33.

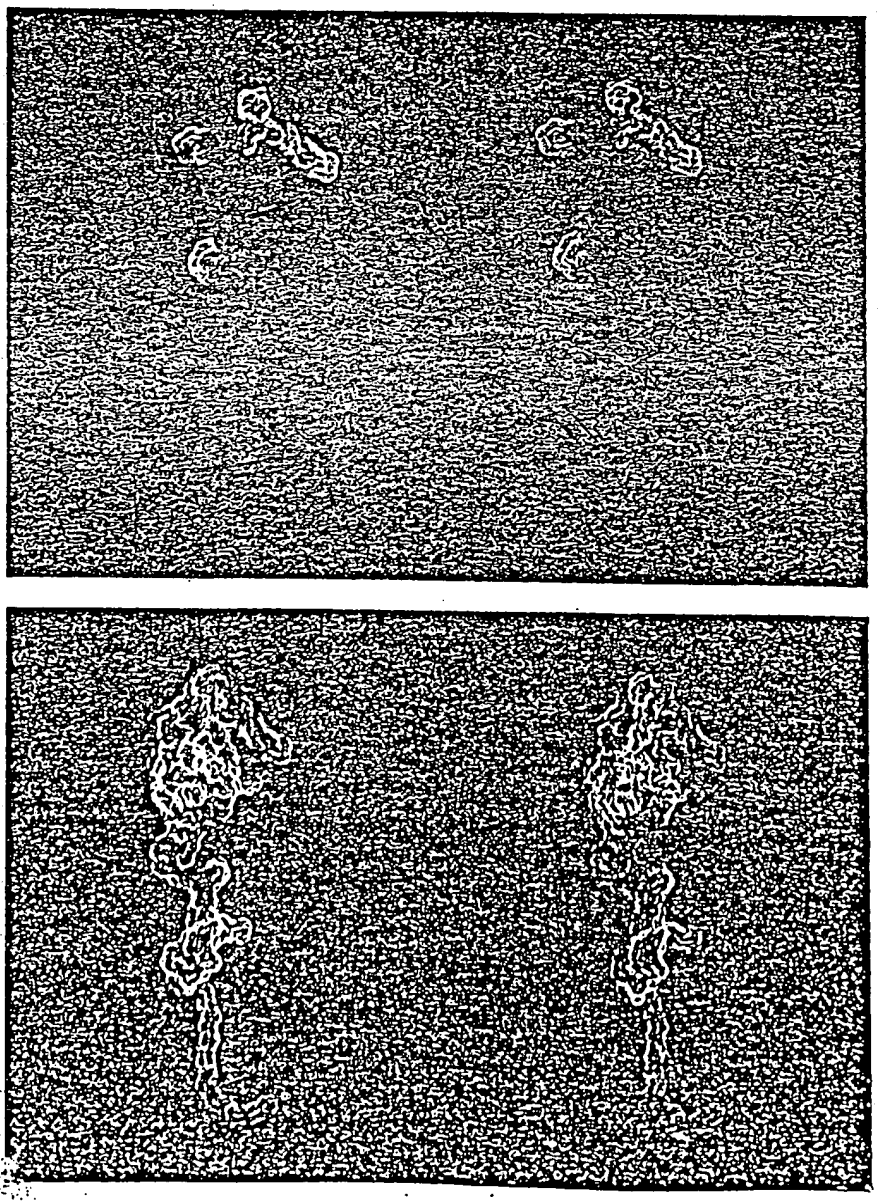

Fig. 2 Antigenic and immunogenic sites (shown in pink) of the influenza virus HA1 molecule. (HA1 shown in green, HA2 shown in blue.) Structure of the molecule based on crystallographic coordinates of Wilson *et al.*[31]. Exposed surfaces based on α-carbon positions using molecular surface computer program of Connolly[74]. *a*, Stereo-pair representing sites eliciting antibodies during the process of infection or immunization with intact virus. *b*, Stereo-pair representing sites against which antibody can be induced by immunization with chemically synthesized peptides.

proteins[26–28]. The conclusions from these studies were clear—the immunogenicity of a protein is less than the sum of the immunogenicity of its pieces[25] with, however, one caveat. The map in Fig. 2 is based on neutralization data and it is possible that during ordinary immunization, antibodies reactive with other parts of the protein are generated but are not scored because they are not neutralizing. (This problem arises because not all antibodies that bind to viral proteins inhibit the infectivity of the virus.) If this were the case then the collection of anti-virus antibodies would have a reactivity pattern much broader than that observed by neutralization studies. This is, however, probably not the case: we have found that high-titred antibody made against the intact haemagglutinin does not react with any of the synthetic peptides[25]. We recently carried out a more compelling demonstration of the exclusion of some reactivities in anti-virus sera by taking advantage of the facts that among various influenza strains there are constant and variable regions of the HA1 and HA2 components of the viral haemagglutinin, and that antibody to the native molecule does not widely neutralize across strains. If an anti-peptide antibody to a conserved region neutralized across strains whereas an anti-virus antibody did not, it would indicate that different immunological specificities are generated during the two types of immunization. We therefore studied antibodies to several peptides from conserved portions of the protein structure and showed that even though the anti-virus antibody has a titre against the homologous strain which is about 100-fold higher than that of the anti-peptide antibodies, only the latter neutralizes across strains (S. Alexander and R.A.L., unpublished). Thus, even during a vigorous immune response against virus, the region represented by these synthetic peptides is not immunogenic; hence, by using peptide immunization one can generate antibody specificities that cannot be obtained in any other way.

Antibody of predetermined specificity in biology

The spread of the use of chemically synthesized peptides to generate antibodies of predetermined specificity is indicated by the number and diversity of experiments recently carried out. The antibodies have been designed for a wide variety of uses.

Detection of proteins predicted from nucleic acid sequences: Anti-peptide antibodies have proved useful in detecting proteins predicted on the basis of nucleic acid sequences to be present in cells. The technology has been particularly successful in discovering DNA and RNA tumour virus proteins implicated in cell transformation. Anti-peptide antibodies have been used to detect the large T antigen of polyoma and SV40 viruses, as well as the cellular transformation-associated protein uniquely expressed in many types of malignant cells[29–31]. Green and Brackmann (personal communication) made an anti-peptide antibody that precipitates the 53,000 molecular weight (53K) protein encoded by the adenovirus EIB transcription unit. As expression of the 53K protein is essential for a fully transformed cell, this antibody together with that made to adenovirus EIA products (see below) should be useful in studying the process of cell transformation. A particularly successful use of anti-peptide antibodies has been in defining the proteins encoded by the oncogenes of the rapidly transforming retroviruses including those of the Moloney sarcoma[32,33], feline sarcoma[34], Rous sarcoma (refs 35, 36 and L. E. Gentry *et al.*, personal communication), avian myeloblastosis[37] and Simian sarcoma virus[38].

Antibodies against functionally active regions of proteins: Peptide hormones are often cleaved from larger precursor proteins, which contain multiple hormones. Anti-peptide antibodies have been used to localize the portions of the 31,000-MW γ-melanocyte-stimulating hormone and 17,500-MW calcitonin precursor which correspond to the functional activities of these two hormones[39,40].

Schaffhauser *et al.* have used anti-peptide antibodies to perturb the functional activity of proteins[41]. The middle T antigen of polyoma virus has been implicated in cell transformation

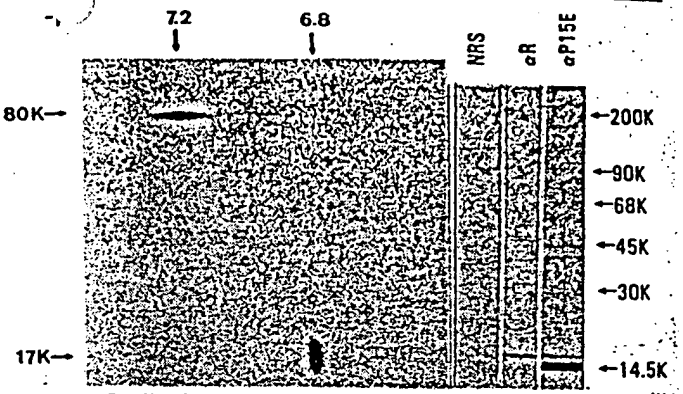

Fig. 3 Studies illustrating the use of anti-peptide antibodies to demonstrate the cleavage of viral protein during virus maturation. Left: immune precipitates of virus infected cells with anti-peptide antibody. Extracts of ^{3}H-leucine-labelled cells were reacted with anti-peptide antibody and the solubilized immune precipitates characterized by two-dimensional gel electrophoresis. The 80K protein is the entire product ($Pr80^{env}$) of the envelope gene and contains N-gp70-15E-C. The 17K protein (pre-15E) was confirmed by radioactive protein sequencing to be the precursor to the 15E anchor protein[44]. Right; immune precipitates of labelled virus showing that amino acids had been cleaved from the C-terminus of pre-15E during virus maturation to yield viral p15E. Virus labelled with ^{35}S-methionine was reacted with normal rabbit sera (NRS); anti-peptide antibody [here called αR to designate that this serum represents antibody against amino acids predicted by right most (3′) possible 15 codons of the viral genome]; and antibody against the mature anchor protein p15E (αP15E). The bulk of the p15E in virus fails to react with αR sera and thus lacks a portion of its C-terminus[44].

and contains a tyrosine kinase activity that predominantly phosphorylates tyrosine 315 of the middle T protein. They synthesized a nonapeptide corresponding to residues 311–319 of the middle T antigen. This antibody not only precipitates middle T in infected cells but inhibits phosphorylation of the protein *in vitro*. Interestingly, the anti-peptide antibody does not react with the middle T antigen when tyrosine 315 is phosphorylated. Shinnick *et al.*[42] have also used anti-peptide antibodies to localize and perturb the functions of enzymes encoded by viral genomes. The retroviral *pol* gene is about twice as big as necessary to encode the 80,000-MW reverse transcriptase[43]. Gene products were sampled by synthesizing peptides at intervals of about 100 amino acids along the predicted *pol* sequence and antibodies against these peptides used to study *pol* gene products in infected cells. Antibodies to peptides predicted from the 5′ end of the gene precipitated an 80,000-MW protein and inhibited the reverse transcriptase activity whereas antibodies to peptides from the middle of the gene precipitated a 40,000-MW protein and inhibited the virus-associated endonuclease activity. Antibodies to peptides from the 3′ end of the gene precipitate a 20,000-MW protein which according to enzyme inhibition studies seems to be protease. Thus, anti-peptide antibodies are useful not only in detecting the protein product of a gene, but also in identifying its function.

Following protein domains: Anti-peptide antibodies have been used in the difficult problem of following the fate of individual protein domains and have shown that the cleavage and removal of the hydrophilic C-terminus from the retroviral membrane anchor protein takes place during virus maturation[44]. The main envelope glycoprotein, gp70, is anchored into the viral membrane through its attachment by disulphide bonds to the membrane spanning proteins, p15E. Antibody against a synthetic peptide corresponding to the C-terminus of the pre-15E portein was made and used to study the fate of this region of the protein during processing leading to virus formation. In infected cells the antibody detected two proteins of molecular sizes of 80K and 17K corresponding, respectively, to the envelope polyprotein precursor containing gp70 and p15E, and one of the cleavage products (pre-15E) (Fig. 3). However, when the proteins of radioactively labelled virus were studied

with the anti-peptide antibody, it could be seen that the C-terminus predicted from the nucleotide sequence was missing from the mature p15E protein (Fig. 3), thus demonstrating its removal during virus maturation. It remains to be seen if cleavage and processing of C-termini are frequent events which will be found in other systems or if they are peculiar to events involved in viral maturation.

Baron and Baltimore[45] and Semler *et al.*[46] used anti-peptide antibodies to follow poliovirus protein domains, by making antibodies to chemically synthesized peptides corresponding to the genome-linked protein (VPg). Since mature poliovirus proteins are generated by a cascade of cleavages starting with a large precursor (NCVPOO), the peptide of interest is located in various positions in the different intermediate cleavage products, ultimately becoming the C-terminus of a 12,000-MW polypeptide from which 22 amino acids are donated to the 5' end of the genomic RNA. In the Baron and Baltimore experiments antibodies raised against the entire 22 amino acids of VPg as well as a peptide corresponding to its 14 most C-terminal amino acids reacted with the peptide when it was present in five different members of the cleavage cascade. As well as providing the solution of a biological problem, these results demonstrate that the reaction of anti-peptide sera with the native protein is relatively independent of the position of the target in the native structure.

Exon usage: A special example of the use of antibodies of predetermined specificity to follow protein domains is in the study of exon usage during gene expression. Shinnick and Blattner[47] used anti-peptide antibodies to follow exon usage in the immunoglobulin-D system. They synthesized peptides uniquely corresponding to the protein encoded by the exons specific for either the secreted or the membrane-bound form of IgD. Each of the anti-peptide antibodies was shown to be specific for one of the alternative forms of IgD and can now be used to follow the fate of these two proteins in cells. These results, again, demonstrate the production of specific reagents which would be difficult to make by other means. Anti-peptide antibodies have also facilitated the study of exon usage in the adenovirus-2EIA transcription unit. EIA encodes functions which both regulate expression of other early viral genes and have a role in cell transformation[48–51]. The EIA region transcript is processed into at least two overlapping mRNAs (12S and 13S) which share 5' and 3' termini and differ by 138 nucleotides. Since the 12S and 13S mRNAs are in the same reading frame and translation is probably initiated at the common first AUG, it has been assumed that the two proteins encoded by these messages have common N- and C-termini and differ by 46 amino acids unique to the middle of the larger protein[52]. Feldman and Nevins prepared antibody to a 13 amino acid long synthetic peptide predicted from the nucleotide sequence to correspond to a hydrophilic portion of the putative 46 amino acids unique to the larger protein and were able to show that this antibody only reacted with the larger of the two proteins encoded in EIA[53]. This antibody should be very helpful in defining the role of the different EIA proteins in transformation and control of transcription.

Anti-'wrong' reading frame antibodies: We prepared anti-'wrong' reading frame antibodies to study frameshift mutations in viral proteins (A. Sen and R.A.L., unpublished). We used the nucleotide sequence of several sarcoma virus transforming genes to predict the protein sequence which would correspond to +1 and +2 frameshifts far enough to the 3' end of the genome so that premature terminations would not occur. Using these antibodies we were able to detect frameshifted proteins in infected cells. Coupled with transfection of genes, such anti-'wrong' reading frame antibodies may be very useful in comparing the fate of wild-type and mutant proteins in the same cells.

Antibody of predetermined specificity in medicine

Small molecules were first used in the design of synthetic vaccines in 1938 when Goebel made antibody to the carbohydrate antigen diazotinized *p*-aminobenzyl cellobiuronide coupled to horse serum globulin[54,55]. Mice immunized with the preparation acquired active resistance to infection with virulent type III pneumococci[54]. Anderer, working from the results of denaturation and cleavage experiments and assuming that the immunogenicity of the tobacco mosaic virus protein depends on retention of conformation, studied a C-terminal hexapeptide coupled to bovine serum albumin. He showed that antibody to the hexapeptide would precipitate and neutralize the virus[56]. Working with phage, Langbeheim and his colleagues synthesized two peptides from the coat protein of MS-2 and made rabbit antiserum against them[57]. They were able to show binding by one of the anti-peptide antibodies. In their experiment, addition of the rabbit anti-peptide antibody was followed by addition of antisera against rabbit immunoglobulin, thus obscuring whether the anti-peptide antibody had any primary neutralizing activity.

Before synthetic vaccines could be realistically designed to combat eukaryotic viruses, two things were required: a method for obtaining protein sequences of relatively scarce viral proteins, and constraints of the actual or perceived need for conformational determinants had to be overcome. Advances by Sanger and Gilbert and their colleagues in nucleic acid sequencing technology solved the problem of obtaining reliable amino acid sequences of viral proteins, and chemical synthesis of peptides allowed production of antibodies not necessarily restricted, to reactivity with conformational determinants within native proteins. Recently, peptides corresponding to influenza HA1 (refs 25, 58), the hepatitis B surface antigen (HB_sAg)[59–62], the foot-and-mouth disease (FMDV) VPI protein[63], and the rabies virus glycoprotein[64], have been synthesized. One interesting result in the HB_sAg system is that peptides differing in only two residues were capable of inducing antibodies in rabbits and chimpanzees which were capable of distinguishing the y and d subtypes of HB_sAg[65]. It appears that synthetic peptides can duplicate serological markers known to be of significance from classical virological and epidemiological studies. In the influenza[66] and FMDV systems[63], synthetic peptides induced neutralizing antibodies. Further studies using antibodies of predetermined specificity should, in turn, improve our understanding of the process of virus neutralization and thus guide the design of useful peptides. One can envisage that the reason for strain variation in viral proteins is due to selective pressures of the immune system, and thus that the variable regions signal areas available for, and sensitive to, antibody binding. Alternatively, one can aim for invariant regions with functional activities. An additional theoretical issue important to the design of new vaccines is that an intact protein when free may fold differently from when it is part of a virus particle and thus may not confront the immune system in such a way as to induce neutralizing antibodies. In these cases, synthetic peptides may offer advantages over purified viral capsid proteins (so-called subunit vaccines) because they can induce anti-virus antibodies which are independent of protein folding and can be directed to neutralizing sites on the virus surface.

Practical considerations

Given a nucleotide sequence, the problem often is which peptide to synthesize. As so many different peptides have been shown to induce antibodies reactive with the native protein, the only essential point is to choose one which contains hydrophilic amino acids and is thus likely to be exposed on the surface of the intact molecule. Also, peptides containing hydrophilic groups are likely to be soluble, making their handling and coupling to carrier molecules easier. A number of computer programs have been used to predict secondary structures[67–69], which indicate regions of proteins located on the surface of the molecule and available to antibody but a simple search of the amino acid sequence for charged residues seems to be equally effective for most studies. In the experiments carried out so far, several different carriers and coupling methods have been used with equally good results, and so this choice does not seem

to be critical as long as an immunogenic carrier is used. However, where cultured cells are used, bovine serum albumin should be avoided as a carrier since it is a component of the growth media to which many cellular proteins bind non-specifically and the presence of antibodies to it can confuse immunoprecipitation studies of radioactively labelled cells.

Another question is that of the titre of an anti-peptide antibody compared with that raised by immunization with the intact protein. The answer, as far as we know, is that sometimes it is greater and sometimes less. However an important point is that by using peptide immunization, one can generate antibody specificities which cannot be obtained in any other way.

The minimum size of the peptide chosen is important and should be larger than six amino acids. We generally synthesize peptides of 15 amino acids. Considerably larger peptides have also proved useful[25] but often do not offer any advantage as one risks the problem that they are more likely to assume a fixed conformation distinct from that of the native molecule.

Anti-peptide antibodies can also be used for immunoaffinity purification of rare proteins[70]. The main advantage of anti-peptide antibodies for immunoaffinity purification of proteins is that elution can be accomplished by excess peptide instead of the usual methods of high salt, low *p*H or chaotropic agents. Since the peptide elution is specific for the antigen–antibody union of interest, the recovered protein is less likely to be contaminated with extraneous proteins which were bound to the column at sites other than the antibody combining site. Also, proteins eluted by peptides are more likely to be recovered in a native state.

Theoretical considerations

The reason anti-peptide antibodies so often react with the native structure seems to be that they can adopt a number of conformations in solution, one of which approximates that adopted in the native molecule. If this is the case anti-peptide antibodies should contain a mixture of reactivities to these various conformations with only a small percentage of them actually reacting with the native structure. There are, however, other theoretical possibilities. One is that a protein molecule perturbed by solvent interactions exists as a statistical ensemble of conformative states with similar backbone structures but with variations in side-chain orientations on the surface of the molecule. In this situation the anti-peptide antibody could either 'lock in' a particular conformation, induce a conformation, or, in fact, mould its own combining site which for similar reasons would itself be somewhat sterically mutable. That at least some of the latter notions may be true is supported by our studies on monoclonal antibodies to chemically synthesized peptides (H. Niman and R.A.L., unpublished). We selected monoclonal anti-peptide antibodies against an influenza virus HA1 peptide (amino acids 75–111) and then looked at what percentage would react with the native protein. Surprisingly, 14 of 27 reacted in an enzyme-linked immunoabsorbent assay (ELISA) and immunoprecipitation assays with intact HA1, a number much too large to be compatible with a simple model of occasional shared conformations between the free peptide and its counterpart in the protein. If these results can be extended and generalized, one will have to think in terms of flexible structures in protein, antibody molecules, or both. Alternatively, both in solution and as part of protein molecules, peptides may spend the bulk of their time in a limited number of comparable conformations presumably representing the state of lowest free energy.

Future prospects

The ability to make antibodies to defined regions of proteins will, no doubt, open the way to a number of biochemical experiments on the structure–function relationships of proteins as well as help in the immunological prevention and perhaps treatment of disease. Consideration of the theoretical basis for the immunogenicity of small synthetic peptides has led to questions about the fluidity of regions of protein molecules in solution, as well as the possibility that antibodies induce shape changes in proteins. The somewhat surprising results concerning the immunogenicity of peptides may lead to a better understanding of the dynamics of protein molecules in solution. The fact that such a high percentage of monoclonal anti-peptide antibodies react with the native protein already suggests that something unexpected is on the horizon.

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